

Unique Nuclear DNA Sequences in the Involved Tissues of Hodgkin's and Burkitt's Lymphomas

(cancer/virus)

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ABSTRACT Previous studies have demonstrated that Hodgkin's, Burkitt's, and other human lymphomas contain particulate elements encapsulating 70S RNA and RNA-dependent DNA polymerase. [3 H]DNA probes endogenously synthesized by these particles were used to demonstrate that the nuclear DNA of the lymphomas contain particle-related sequences that cannot be detected (less than 1/20th of a copy per genome) in the DNA of normal cells. This result agrees with our earlier findings in human leukemias. The data are inconsistent with any etiologic concept that invokes germ-line transmission of at least one complete copy of the particulate information associated with the malignancy. The unique sequences found in the nuclear DNA of Burkitt's and Hodgkin's tissues are related to each other but not to the DNA of the Epstein-Barr virus.

We have been searching for evidence in human neoplasias of possible viral agents. First we used appropriate murine RNA tumor viruses (i.e., mouse mammary tumor virus and Rauscher leukemia virus) to generate highly radioactive DNAs complementary to the viral RNAs. Molecular hybridizations with these radioactive DNAs revealed that human adenocarcinomas of breast (1, 2), leukemias (3), sarcomas (4), and lymphomas (5-7) contain RNA molecules possessing a small but significant homology to the RNAs of the tumor viruses that cause the corresponding malignancies in mice. Using the simultaneous detection test (8, 9) we demonstrated that the RNA identified in these human cancers was 70 S in size and encapsulated with an RNA-directed DNA polymerase in a particle of density between 1.16 and 1.19 g/ml (10-13). The DNAs synthesized endogenously by these particles possessed complementarity to the RNAs of the analogous murine tumor viruses.

Collectively, these experiments documented the existence in various human neoplastic tissues of particulate elements possessing four features diagnostic of animal RNA tumor viruses. Here we focus on the question of whether normal cells contain in their genome at least one complete copy of the information necessary and sufficient for particle production. We have previously considered this problem in human leukemias in which the methodology had already been developed (10) to separate the particles. These were then used to generate the [3 H]DNA-labeled DNA probes required to search for the corresponding sequences in nuclear DNA. The

data obtained (14) with eight leukemic patients demonstrated that the DNA of leukemic cells contained particle-related sequences that could not be detected in the leukocyte DNA of normal individuals. This conclusion was further strengthened and made biologically more precise by showing (15) that the leukemic members of two sets of identical twins contained particle-related sequences in their leukemic cell DNAs that could not be detected in the leukocytes of their healthy siblings. These findings imply that the additional specific information found in the DNA of the leukemic individuals must have been inserted subsequent to fertilization. Thus, the outcomes argue against any etiologic concept that invokes vertical transmission through the germ line of the particle-related information found uniquely in the DNA of leukemic cells.

We wanted to see whether these findings hold also for some other human neoplasias in which similar particles have been identified (12, 13). In this paper we describe experiments with Hodgkin's and Burkitt's lymphomas. The data show that the nuclear DNA of both types of lymphomas contain unique sequences that are not detectable in normal cellular DNA.

METHODS

Preparation of [3 H]DNA. Ten grams of lymphoma tissues were disrupted with a Silverson homogenizer at 4° in 2 volumes of TNE buffer (0.01 M Tris·HCl, pH 8.3-0.15 M NaCl-0.01 M EDTA). The suspension was centrifuged at $4000 \times g$ for 10 min at 4°, and the supernatant was recentrifuged at $10,000 \times g$ for 10 min at 4°. The resulting supernatant fluid was then layered on a 13-ml column of 20% glycerol in TNE and centrifuged at $98,000 \times g$ for 1 hr at 4° in a SW-27 rotor (Spinco). The pellet was resuspended in TNE and layered on a linear gradient of 15-55% sucrose in TNE and spun in a SW-27 (Spinco) rotor at 4° for 210 min. The gradient was dripped from below, and the density region between 1.15 and 1.19 g/ml was collected and pooled. After dilution with TNE, this region was pelleted at $100,000 \times g$ for 1 hr. The resulting pellet was resuspended in 1.0 ml of 0.01 M Tris·HCl, pH 8.3, brought to 0.15% Nonidet P-40 (Shell Chemical Co.), and incubated at 0° for 15 min. DNA was synthesized in an RNA-instructed DNA polymerase reaction mixture (final volume 2.0 ml) containing: 200 μ mole of Tris·HCl, pH 8.3, 80 μ mol of NaCl, 24 μ mol of MgCl₂, 400 μ mol each of dATP, dGTP, dCTP, and 200 pmol of [3 H]dTTP (50 Ci/mmol). Actinomycin D (100 μ g/ml) was added to inhibit DNA-instructed DNA synthesis. After incubation at 37° for 15 min, the reaction was adjusted to 0.2 M

Abbreviation: C₀t, product of concentration of nucleotide sequences of DNA and time of incubation.

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NaCl and 1% sodium dodecyl sulfate. After phenol extraction, the aqueous phase was subjected to Sephadex G-50 chromatography and the DNA region of the column was pooled and collected. The [3 H]DNA product was then adjusted to 0.4 M NaOH, incubated at 37° for 6 hr to destroy RNA, and then neutralized.

Preparation of Nuclear DNA. The nuclear pellet ($1000 \times g$ for 10 min) was resuspended in TNE and lysed by addition of sodium dodecyl sulfate to a concentration of 1% in the presence of 1 M NaClO₄. After repeated extraction with phenol-cresol and chloroform-isoamyl alcohol, DNA was spooled out of solution. The DNA was redissolved in 3 mM EDTA and sheared to 6-8 S as measured in a 10-30% glycerol gradient. Shearing was accomplished with a Bronwill Biosonic IV sonifier using the microtip at maximum power for two 30-sec intervals. The sample was then incubated with 0.4 M NaOH for 16 hr at 37°. After neutralization, DNA was precipitated with alcohol and redissolved in 3 mM EDTA. The DNA preparations had an A_{260}/A_{280} ratio greater than 1.9 and renatured more than 80%, as measured by hydroxyapatite chromatography.

Annealing Conditions. Annealing reactions contained >60 A_{260} units of nuclear DNA, 0.1-1.0 pmol of [3 H]DNA, and 0.1% sodium dodecyl sulfate at a final DNA concentration of >15 mg/ml. The reaction was brought to 100° for 10 min and 0.04 mmol of NaCl was added. The reaction mixture was then incubated at 65°, and stopped by addition of 4 ml of 0.01 M NaH₂PO₄, pH 6.8. The sample was then passed over a hydroxyapatite (16, 17) column of 2-ml bed volume/mg DNA at 60°. The column was washed with 16 ml of 0.01 M phosphate buffer at 60° and then 20 ml of 0.15 M NaH₂PO₄, pH 6.8, at 60°, 80°, 88°, and 95°. Fractions of 4 ml were collected, and the [3 H]DNA counts in each fraction were assayed by scintillation counting in 10 ml of Aquasol (NEN). The method identifies unpaired strands that elute at 60° and poorly paired duplexes that disassociate at 80°. Only the duplexes disassociating and eluting at 88°-95° are counted here as hybridized.

Recycling of Probe on Normal DNA to Remove Normal Sequences. The reactions were done with normal DNA as above and the eluates were collected in 1-ml fractions at 60°. The peak fractions were combined and the resulting pools were passed over a Sephadex G-50 column of 10-ml bed volume. The DNA regions were collected and the DNA was precipitated with 2 volumes of 100% ethanol. The material eluted at 60° was used as the recycled product unable to hybridize to normal DNA.

RESULTS

RNA of the human leukemic particles shared sequences with the DNA of normal cells (14), a feature also exhibited (18-25) by some animal RNA tumor viruses and the normal DNA of their putative indigenous hosts. Sequences common to both normal DNA and the [3 H]DNA synthesized endogenously by the particle system can be removed by exhaustive hybridization to a vast excess of normal DNA followed by hydroxyapatite chromatography to separate duplexes from unpaired [3 H]DNA. The residue of unpaired [3 H]DNA can then be used to obtain an answer to the question: "Does the DNA of the neoplastic cells contain sequences not found in normal cells?"

These considerations dictate the following strategy for the experiments to be described: (1) Isolate the particles encap-

sulating 70S RNA and the RNA-directed DNA polymerase from human lymphoma specimens; (2) use the particle fraction to endogenously synthesize [3 H]DNA in the presence of a high concentration (100 μ g/ml) of actinomycin D to inhibit host or viral DNA-directed DNA synthesis; (3) purify the [3 H]DNA by Sephadex chromatography and hydroxyapatite; (4) remove the [3 H]DNA sequences shared with normal DNA by exhaustive hybridization in the presence of vast excess of normal DNA followed by hydroxyapatite chromatography to separate paired from unpaired [3 H]DNA; and (5) test the unpaired residue for specific hybridizability to lymphoma DNA.

[3 H]DNA probes were synthesized from four Burkitt's tumors, three Hodgkin's disease specimens, and one lymphosarcoma. In all instances the lymphoma [3 H]DNA probe hybridized 35-40% to normal spleen nuclear DNA, in agreement with our experience with human leukemias (14).

The sequences shared with normal nuclear DNA were then removed by exhaustive annealing of the [3 H]DNA with normal spleen DNA in vast excess. In this step, annealing reactions were set up to contain 60 A_{260} units of normal cellular DNA per 0.1 pmol of [3 H]DNA (1000 cpm) corresponding to a 100- to 1000-fold excess of the relevant sequences in normal DNA. The annealing was continued for C_{ot} values (26) in excess of 10,000, and the unpaired [3 H]DNA was recovered by hydroxyapatite chromatography. These should no longer contain sequences complementary to those found in normal DNA, and exclusive hybridizability of such recycled [3 H]DNA to lymphoma DNA would then establish that the genome of lymphoma cells contains specific sequences not present in normal DNA.

We illustrate a representative elution result with recycled [3 H]DNA made with the particles derived from Hodgkin's and

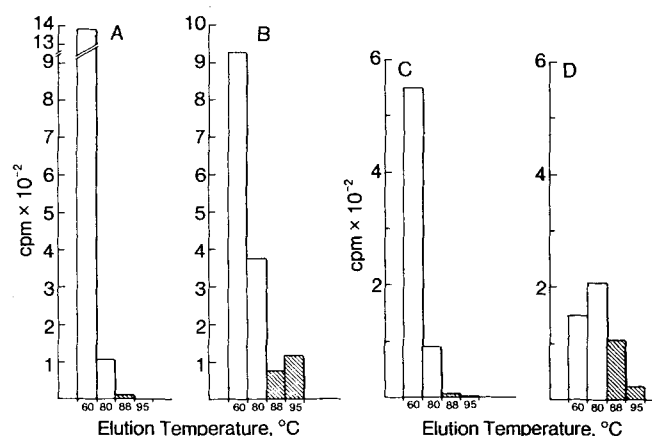


FIG. 1. Hydroxyapatite elution profiles of hybridization reactions of recycled Hodgkin's disease [3 H]DNA to nuclear DNA from (A) normal human spleen, (B) a spleen involved with Hodgkin's disease; and recycled Burkitt's lymphoma [3 H]DNA to nuclear DNA from (C) normal human spleen and (D) Burkitt's tumor. Hybridization reactions contained 2000 cpm of recycled [3 H]DNA and 2 mg of nuclear DNA (concentration >15 mg/ml). Hybrid formation was analyzed by hydroxyapatite chromatography at a phosphate buffer (pH 7.2) eluent concentration of 0.15 M. Five fractions of 4 ml were collected at each of the four temperatures, and the [3 H]DNA counts in each fraction were assayed by scintillation counting in 10 ml of Aquasol (NEN). All radioactive samples were counted for 10 min to assure the necessary accuracy.

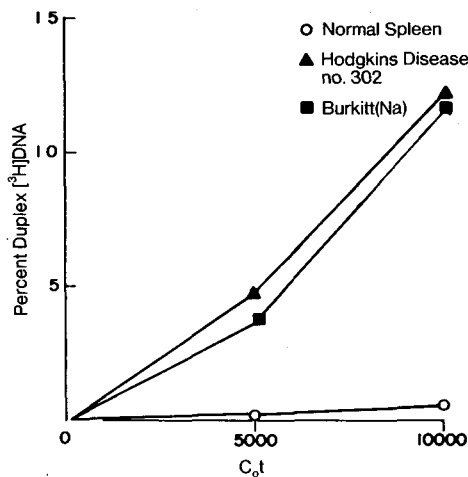


FIG. 2. Hybridization of recycled Hodgkin's disease no. 302 [^3H]DNA to nuclear DNA isolated from normal spleen (O—O), Hodgkin's disease no. 302 (\blacktriangle — \blacktriangle), and Burkitt's lymphoma (Na) (\blacksquare — \blacksquare). Hybridization reactions in which duplex formation was analyzed at multiple C_{ot} values contained proportionately increased amounts of recycled [^3H]DNA probe and nuclear DNA. Equal aliquots were removed from the hybridization vessel at each C_{ot} value, and hybrid formation was analyzed by hydroxyapatite chromatography (see legend to Fig. 1).

Burkitt's lymphoma. In each case the recycled [^3H]DNA is rechallenged against normal DNA and against the nuclear DNA of the neoplastic tissue from which the particles were derived. In neither case do the recycled probes enter into complexes with normal DNA that are stable above 80° (Fig. 1). However, each probe forms well-paired duplexes with the DNA from the tumor of origin.

Fig. 2 shows similar outcomes at two different C_{ot} values. Recycled Hodgkin's disease [^3H]DNA is challenged with nuclear DNA from normal spleen, Hodgkin's disease spleen, and from Burkitt's lymphoma. The input counts for each C_{ot} point were 1500–2000 cpm. Only those duplexes disassociating and eluting above 88° are counted here as stably hybridized. Few, if any, stable complexes are formed with normal DNA; over 10% of the input [^3H]DNA pairs with both Hodgkin's and Burkitt's disease nuclear DNA. Although the probe was made with Hodgkin's disease particles, clear evidence is seen of homology with sequences in the nuclear DNA of Burkitt's lymphoma. That the converse also obtains is shown in Fig. 3, in which recycled Burkitt's [^3H]DNA is challenged with various nuclear DNAs, including one from Hodgkin's disease to which it does hybridize. The Burkitt's [^3H]DNA complexes to nuclear DNA from two Burkitt's tumors and to nuclear DNA from a pool of Burkitt's lymphoma cell lines (EB-2 and EB-3). In contrast, no significant hybridization was detectable when the same probe was challenged with normal spleen DNA, infectious mononucleosis spleen DNA, or against NC-37 cell DNA, a "normal" leukocyte cell line containing multiple copies of the Epstein-Barr virus genome (27).

Table 1 summarizes all of our findings with the recycled lymphoma [^3H]DNA probes annealed with nuclear DNA from normal and malignant tissues. Normal DNA is unable to form significant amounts of stable complexes with the [^3H]DNA. In all instances, the lymphoma [^3H]DNAs hybridized to the nuclear DNA of the original lymphoma types from

which the particles were obtained and used to generate the DNA probes.

With one exception, all of the Burkitt's and Hodgkin's disease [^3H]DNA cross-hybridized with each other's nuclear DNA. This outcome suggests that particle-related information found in these two lymphomas share sequences in common in agreement with our earlier finding that all types of lymphomas, including Burkitt's and Hodgkin's contain particulate RNA homologous to Rauscher leukemia virus RNA. One would predict then that [^3H]DNAs made from Burkitt's and Hodgkin's particles would hybridize with the RNA from both neoplastic tissues (Fig. 4). The recycled [^3H]DNA synthesized by Burkitt's particles does hybridize to the RNA from a Hodgkin's spleen (Fig. 4A) and not to the RNA from a normal spleen (Fig. 4B). The converse of hybridizing recycled [^3H]DNA generated by Hodgkin's particles to the RNA of Burkitt's tumors has been done, but is not detailed here. Recycled [^3H]DNA synthesized by particles from lymphoma hybridized to Rauscher leukemia virus RNA (Fig. 4C) but not (Fig. 4D) to the RNA of the unrelated mouse mammary tumor virus.

DISCUSSION

Whether our inability to detect the lymphoma-specific sequences in normal DNA means that the latter contains less than one copy per genome equivalent depends on the sensitivity of the detection and the multiplicity of the specific sequences in the malignant DNA. The specific activity of the [^3H]TTP used, when corrected for counting efficiency and

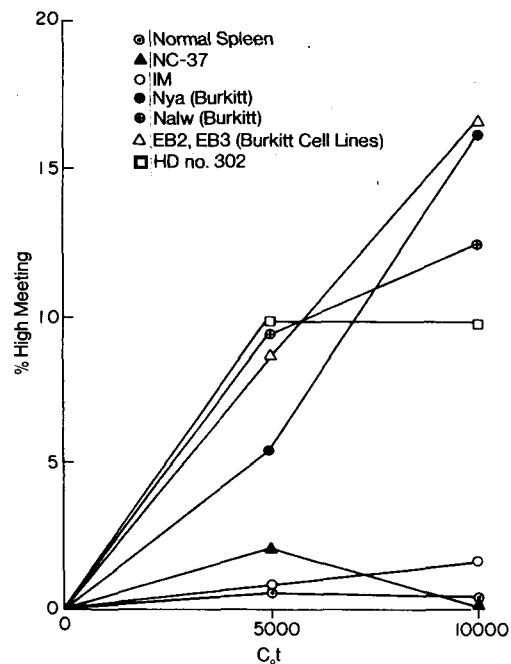


FIG. 3. Hybridization of recycled Burkitt's lymphoma (Nya-T) [^3H]DNA to nuclear DNA isolated from normal human spleen (O—O), a "normal" lymphoblastoid cell line containing Epstein-Barr virus (NC-37) (\blacktriangle — \blacktriangle), Burkitt's lymphoma (Nya) tumor (\bullet — \bullet), Burkitt's lymphoma (Na) tumor (\oplus — \oplus), a pool of Burkitt's lymphoma cell lines, (EB-2 and EB-3) (\triangle — \triangle), and a Hodgkin's disease no. 302 spleen (\square — \square). Hybridization reaction conditions and duplex formation analysis are as described in the legend to Fig. 2. Only duplexes disassociating and eluting at 88° and 95° are counted as high melting.

composition of the product, results in a specific activity of 3×10^7 cpm/ μ g or 10,000 cpm/pmol. Since actinomycin D was included in all syntheses, the single-stranded DNA transcripts would be expected to represent a substantial portion of the particle RNA sequence (28). If the normal DNA contained one copy of the probe per genome equivalent, the 300 μ g of DNA used in our hybridizations would contain about 3×10^{-3} μ g of the relevant sequences, and the specificity of our probe would permit us to detect at saturation 1/1000 of a single copy per genome. The question of multiplicity of the lymphoma-specific sequences in the tumor DNA must be considered in comparing the results obtained with normal DNA. Thus, if the lymphoma-specific sequences were represented 100 times per malignant genome, and if the annealings were done at saturation so that all the multiple copies were being counted, then one would have to demonstrate that normal DNA contains less than 1/100 that found in malignant DNA.

TABLE 1. Hybridization of recycled [3 H]DNA probes synthesized by human particles with nuclear DNA obtained from normal and tumor tissues

Recycled [³ H]DNA probe	nDNA hybridized	High melting	
		Cpm	%*
Burkitt lymphoma			
(Ny)	Burkitt (Ny)	209	9.17
(Ny)	Normal spleen	24	1.83
(Na)	Burkitt (Ny)	52	11.12
(Na)	Normal spleen	0	0.00
(NaT)	Burkitt (NaT)	197	15.43
(NaT)	Burkitt (Nya)	30	11.09
(NaT)	H.D. no. 302	40	11.64
(NaT)	Normal spleen	3	0.56
(NyaT)	Burkitt (Nya)	28	16.05
	Burkitt (Na)	21	12.33
	H.D. no. 302	29	9.80
	EB-2 and EB-3 (Burkitt cell lines)	26	16.60
	NC-37 ("normal" leukocyte cell line)	0	0.00
	Infectious mononucleosis spleen	2	1.65
	Normal spleen	1	0.39
Hodgkin's disease			
no. 302	H.D. no. 302	63	12.12
no. 302	Burkitt (Na)	28	11.72
no. 302	Normal spleen	7	0.47
no. 304	H.D. no. 304	106	4.87
no. 304	Burkitt (Ny)	56	5.97
no. 304	Normal spleen	4	0.66
no. 306	H.D. no. 306	119	9.60†
no. 306	Burkitt (Ny)	5	0.82†
no. 306	Normal spleen	7	0.83†
Lymphosarcoma			
no. 300	H.D. no. 302	154	14.2
no. 300	Normal spleen	16	1.8

nDNA, nuclear DNA; H.D., Hodgkin's disease.

* Values represent percentage of counts dissociating and eluting at 88°–95° at a C_0t of 10,000 unless otherwise noted. Scintillation counting was performed for 10 min in all cases to achieve the necessary accuracy and are expressed as corrected for background.

† $C_0t = 5000$.

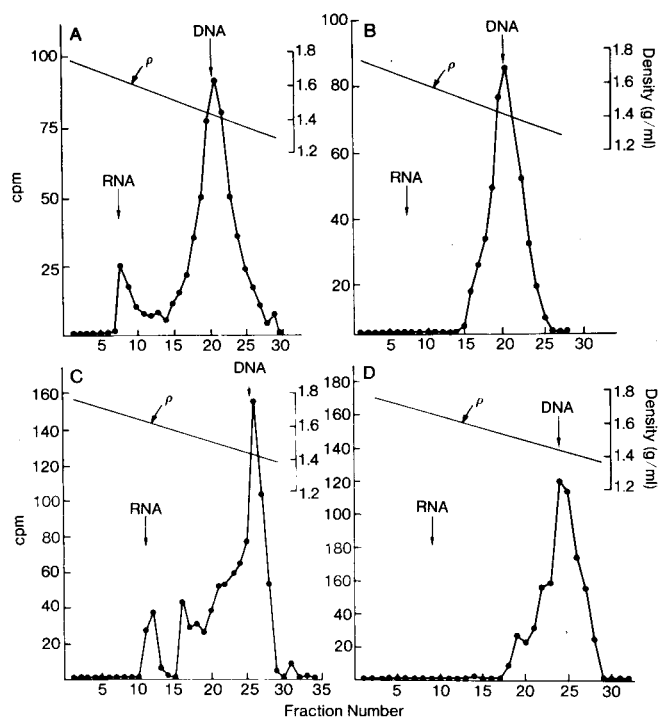


FIG. 4. Cs_2SO_4 equilibrium density gradient centrifugation of recycled Burkitt's lymphoma (Na) [^3H]DNA hybridized to (A) Hodgkin's disease no. 304 polysomal RNA (pRNA) and (B) normal spleen RNA, and of recycled lymphosarcoma [^3H]DNA hybridized to (C) Rauscher leukemia virus 70S RNA and (D) mouse mammary tumor virus 70S RNA. pRNA was isolated from the Hodgkin's and normal spleens by disruption with a Silverson homogenizer at 4° in 2 volumes of 5% sucrose in TNM buffer (0.01 M Tris-HCl, pH 7.4–0.15 M NaCl–2 mM MgCl_2). The suspension was centrifuged at $15,000 \times g$ for 40 min at 0°. The supernatant fluid was then layered on 20 ml of 25% sucrose in TNM buffer and centrifuged for 180 min at $180,000 \times g$ in a Spinco 60 Ti rotor. The pellet (P-180) was resuspended in TNM and 1% sodium dodecyl sulfate, and the RNA was extracted twice with an equal volume of cresol-phenol (pH 8.4). Nucleic acid was precipitated from the aqueous phase by addition of two volumes of ethanol and $1/10$ volume of 4 M LiCl. Recycled [^3H]DNA (2000 cpm reaction) was first incubated at 80° for 10 min in 50% formamide to denature the DNA. After quick chilling to 0°, 400 μ g of pRNA or 1 μ g of purified 70S viral RNA was added. The annealing mixtures were brought to 0.4 M NaCl–50% formamide in a total volume of 100 μ l and incubated at 18 hr at 37°. After incubation the reaction mixtures were added to 5.5 ml of 5 mM EDTA, mixed with an equal volume of saturated Cs_2SO_4 to yield a starting density of 1.52, and centrifuged at 44,000 rpm in a 50 Ti rotor (Spinco) for 60 hr at 15°. 0.4-ml fractions were collected and assayed for trichloroacetic acid-precipitable radioactivity.

However, complications due to multiplicity are minimized by the quantitative aspects and design of the experiments. First, the C_0t values required to detect the lymphoma-specific sequences by the recycled [^3H]DNA probes insure that we are dealing not with highly reiterated sequences, but rather with sequences possessing a multiplicity close to unity.

Examination of the results with normal spleen DNAs (Figs. 2 and 3) show that they contain less than 1/20th of an equivalent found in the DNAs from Burkitt's and Hodgkin's tissue at a C_0t value of 10,000. Indeed, one of the normal spleen

DNA was carried out to a C_{ot} value of 50,000 with no evidence of sequences complementary to a Hodgkin's [^3H]-DNA probe.

One is led to conclude that a normal genome contains less than 1/20th of an equivalent of the DNA sequences found in lymphoma DNA and homologous to the DNA synthesized by the particles found in these neoplastic diseases. Any value clearly less than unity for normal DNA argues against any concept (29) that postulates germ-line transmission of a complete set of the information contained in the particulate elements found in the tumor tissue.

Several other features emerged from this study. The particle-related sequences found in Burkitt's and Hodgkin's lymphomas possess sequences in common, suggesting a relation between their respective particles, and in accord with our earlier findings (5, 6, 12, 13) that Hodgkin's and Burkitt's particles both share sequences with the murine Rauscher leukemia agent. Further, in view of the previous association of the Epstein-Barr virus with Burkitt's disease (30-35) and the non-neoplastic infectious mononucleosis (36, 37), it is of interest that the leukocyte DNA of patients with infectious mononucleosis were devoid of Burkitt's particle-related sequences, indicating that these latter sequences are specific for neoplastic tissues. The fact that the particle-related sequences in Hodgkin's and Burkitt's tumors are homologous adds further weight to this conclusion. Finally, the fact that cells (e.g., NC-37, infectious mononucleosis leukocytes) carrying multiple copies of the DNA of Epstein-Barr virus do not complex with the [^3H]DNA synthesized by either Hodgkin's or Burkitt's particles proves that these sequences have no relation to DNA of Epstein-Barr virus.

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